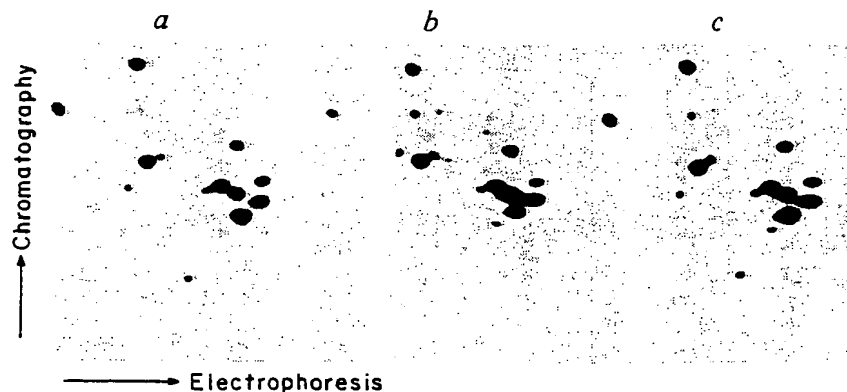


Exhibit 6

Fig. 5 Brain 4.1 and synapsin I have very similar peptide maps. *a*, Brain 4.1; *b*, synapsin; *c*, a mixture of brain 4.1 and synapsin. Peptide maps of bovine brain 4.1 and bovine synapsin I were prepared by the method described in ref. 27.



synaptic vesicles and the plasma membrane. However, spectrin has also been identified as an axonally transported protein, sometimes associated with membrane-bounded organelles³⁵. Thus, an additional or alternative role for spectrin and synapsin might encompass linkage (or regulation of linkage) of synaptic vesicles to cytoskeletal elements. Such cytoskeletal elements need not be only actin. Although erythrocyte 4.1 seems to enhance the binding of brain spectrin to actin³⁶, our preliminary results have not so far revealed such a role for synapsin. However, synapsin may bind microtubules (unpublished data), which are also present in synaptosomes³¹.

Erythrocyte 4.1 is able to bind at least one transmembrane protein. Binding of synapsin to a transmembrane protein of the synaptic vesicle may be of importance in regulation of neurotransmitter secretion, as synapsin is released from its membrane binding site on phosphorylation of synapsin by cAMP- or Ca^{2+} /calmodulin-dependent protein kinases²⁶, which phosphorylate synapsin in response to hormonal or electrical stimulation of nerves³⁷.

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Expression of human growth hormone-releasing factor in transgenic mice results in increased somatic growth

Robert E. Hammer*, Ralph L. Brinster*, Michael G. Rosenfeld†, Ronald M. Evans‡ & Kelly E. Mayo‡

* Laboratory of Reproductive Physiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

† School of Medicine, University of California, San Diego, California 92023, USA

‡ Molecular Biology and Virology Laboratory, The Salk Institute for Biological Studies, PO Box 85800, San Diego, California 92138, USA

The neurohumoral regulation of growth hormone secretion is mediated in part by two hypothalamic peptides that reach the anterior pituitary via the hypothalamo-hypophyseal portal blood system¹. Somatostatin inhibits the release of growth hormone², whereas growth hormone-releasing factor (GRF) positively regulates both growth hormone synthesis^{3,4} and secretion^{5,6}. Two forms of human GRF, 40 and 44 amino acids long, have been characterized from extra-hypothalamic tumours^{7,8} as well as from the hypothalamus⁹. Analysis of human GRF complementary DNA^{10,11} and genomic¹² clones indicates that the GRF peptides are first synthesized as a 107- or 108-amino-acid precursor protein. To examine the physiological consequences of GRF expression, we have established strains of transgenic mice containing a fusion gene including the promoter/regulatory region of the mouse metallothionein-I (MT-I) gene¹³ and the coding region of the human GRF gene¹². We report that expression of the human GRF precursor protein in these animals results in measurable levels of human GRF and increased levels of mouse growth hormone in plasma and accelerated growth rates relative to control littermates. These results demonstrate a direct role for GRF in the positive regulation of somatic growth. Unexpectedly, female transgenic mice carrying the MT-GRF fusion gene are fertile, in contrast to female transgenic mice expressing human or rat growth hormone, which are generally infertile. These transgenic mouse strains should provide useful animal models for the study of several types of human growth disorders.

It was shown previously that the hormonal cascade controlling somatic growth could be manipulated by the germline introduction of foreign genes. Expression of either rat¹⁴ or human¹⁵ growth hormone (GH) in transgenic mice results in increased growth rates, giving rise to large animals. Because GRF is believed to be an important physiological regulator of growth hormone in animals, we sought to analyse further its role in growth control by creating strains of animals that express supraphysiological levels of GRF. Neither rat nor human growth hormone genes are themselves expressed in transgenic animals^{16,17}; this deficiency has been overcome by fusing these structural genes to an expressed heterologous gene promoter.

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Exhibit 6

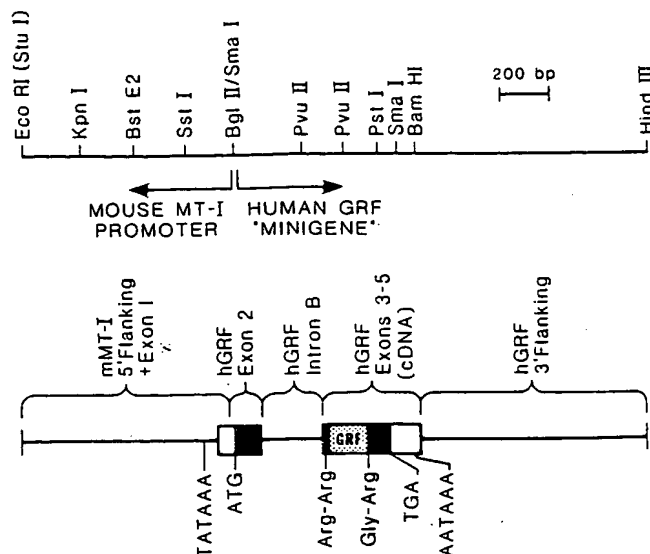


Fig. 1 Structure of the mouse metallothionein-I/human growth hormone-releasing factor fusion gene. The upper portion of the figure is a restriction map for common 6-bp cutters. *BglII/SmaI* indicates the site of the fusion between the mouse MT-I promoter and the human GRF minigene. The lower portion of the figure is a schematic representation of the 2.5-kb *EcoRI-HindIII* fragment of DNA used for microinjection. Open boxes represent 5' and 3' untranslated exon regions, and shaded boxes represent the coding exon regions. The derivations of various parts of the fusion gene are indicated. The fusion occurs in the 5' untranslated regions of both genes. The large exon labelled 3-5 was constructed by replacing part of the human GRF gene with a human GRF cDNA, effectively eliminating intron C (2.4 kb) and intron D (3.0 kb) of the human GRF gene¹². Consensus sequences involved in transcription initiation, translation initiation and termination, polyadenylation, and proteolytic processing of the GRF peptide from its precursor are shown.

The mouse metallothionein-I gene promoter offers several advantages for this type of approach; it is expressed at fairly high levels in most mouse tissues¹⁸, it can be regulated by heavy metals¹⁸, and both expression and regulation are retained in gene transfer experiments¹⁹⁻²¹. Because of the possibility that the human GRF gene, like the human growth hormone gene, might not be expressed in transgenic animals, we initially chose to use the heterologous mouse MT-I promoter to express human GRF.

The structure of the mouse MT-I/human GRF fusion gene construct used (referred to as MT-GRF) is shown in Fig. 1. A 770-base pair (bp) fragment of the mouse MT-I gene, including sequences responsible for metal-inducibility and transcription initiation, was fused to a human GRF minigene which includes the entire coding region of the GRF precursor protein. The human GRF minigene was created by combining cDNA and genomic clones such that the 10-kilobase (kb) human GRF gene, which normally includes five exons¹², has been reduced to <1 kb and retains a single intron of 230 bp (see Fig. 1). Analysis of human GRF cDNA clones suggests that the GRF precursor protein can consist of either 107 or 108 amino acids, differing in the presence or absence of serine 103 (ref. 11). DNA sequence analysis of the human GRF gene¹² indicates that this difference may be explained by alternative RNA processing. The cDNA used to construct the MT-GRF fusion gene should encode only the 108-amino-acid form of the GRF precursor protein¹⁰. To determine whether the MT-GRF fusion gene could be expressed and regulated correctly, it was introduced into cultured mouse fibroblast cells using CaPO_4 -mediated DNA transfection²² and co-selection for neomycin resistance conferred by the vector pSV2-neo²³. Several stable cell lines that were generated in this manner express a MT-GRF fusion mRNA of the expected size whose abundance is increased by metal treatment, and accumu-

Table 1 Expression of the MT-GRF fusion gene in transgenic mice

Mouse	No. of gene copies per cell	Liver GRF mRNA (relative amount)	Plasma hGRF (ng ml ⁻¹)	Plasma mGH (ng ml ⁻¹)	Growth (ratio)
♂760-5	<1	3	24	1,051	1.35
♂762-3	2	0	<10	21	0.93
♀762-5	4	63	26	141	1.33
♀765-2	2	102	207	415	1.24
♂800-1	1	0	<10	149	1.12
♀800-8	1	3	14	402	1.05
♂801-3	3	0	<10	38	0.97
♀801-5	8	38	99	809	1.35
♂801-9	1	4	20	541	1.42
♀802-3	5	1	45	913	1.51
♀803-4	10	118	263	1,095	1.41
♀803-5	1	5	24	166	1.45
♂803-6	10	16	50	302	1.24
♀803-7	20	*	*	*	1.36

A 2.5-kb *EcoRI-HindIII* fragment containing the MT-GRF fusion gene was microinjected into the male pronucleus of fertilized F₂ hybrid mouse eggs and the eggs implanted into the oviducts of pseudopregnant recipients²⁴. At weaning, DNA was isolated from a piece of tail and analysed for the fusion gene by dot-blotting using a human GRF cDNA as probe¹⁰. Positive animals were re-analysed by Southern DNA blotting and copy number determined by comparison with a standard curve generated from known amounts of MT-GRF plasmid DNA. Positive animals were maintained on water containing 25 mM ZnSO₄. RNA was isolated following partial hepatectomy and analysed by Northern blotting using a human GRF cDNA probe. Autoradiograms were densitometrically scanned to determine relative RNA amounts. Plasma GRF and GH were determined by radioimmunoassay of serum samples from animals at 9 weeks old. The GH radioimmunoassay was able to detect 16 ng ml⁻¹ serum GH (two control animals had 16 and 46 ng ml⁻¹ GH) and the GRF radioimmunoassay was able to detect 10 ng ml⁻¹ serum GRF (two control animals had <10 ng ml⁻¹ GRF). The growth ratio was determined at 9 weeks of age and represents a comparison with age- and sex-matched littermates.

* 2803-7 died prematurely. Although we were able to measure liver GRF mRNA, the amount could not be quantitated because of RNA degradation in the postmortem liver. Plasma GRF and GH levels could not be determined accurately for the same reason.

late radioassayable human GRF (hGRF) in the culture medium (K.E.M., unpublished results).

The MT-GRF fusion gene (~1,600 molecules) was microinjected into the male pronucleus of 350 F₂ hybrid eggs (obtained by mating C57BL/6 × SJL hybrid adults) and the eggs were transferred into the oviducts of pseudopregnant recipients as described elsewhere²⁴. Fifty-nine animals developed from the microinjected eggs; at the time of weaning, DNA was isolated from a piece of tail taken from these animals. The presence of the foreign MT-GRF gene was detected by hybridization to a human GRF cDNA clone¹⁰ using conditions in which cross-hybridization with the mouse GRF gene is negligible. Fourteen animals carried the MT-GRF fusion gene; these animals were maintained on water with 25 mM ZnSO₄ to enhance expression of the fusion gene¹⁵. Table 1 summarizes the expression of the MT-GRF fusion gene in these 14 transgenic mice; 11 of the 14 animals express the MT-GRF messenger RNA in the liver, a major site of metallothionein expression¹⁸. The amount of the fusion mRNA in the livers of these mice varies by as much as 100-fold, and does not seem to be strongly correlated with the number of copies of the fusion gene in each animal. Animals expressing the fusion gene have measurable levels of human GRF in their serum, and have increased levels of serum growth hormone (Table 1). Ten of the mice showed significant increases in growth at 9 weeks old, and were 25-50% larger than control littermates. At maturity, some of the MT-GRF animals were nearly twice the size of control animals. Examination of the data in Table 1 reveals that there is no strong correlation between GRF levels, GH levels and growth.

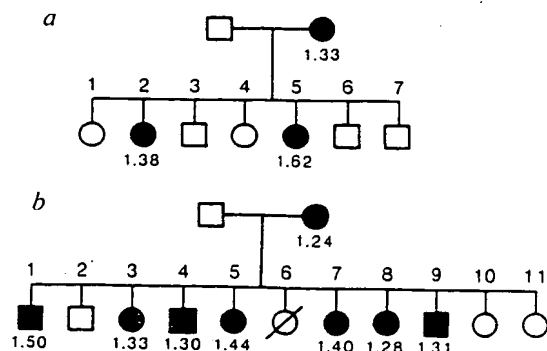


Fig. 2 Pedigree analysis of MT-GRF transgenic mice. Mice that carry the MT-GRF fusion gene (determined by DNA blotting of tail snips) are indicated by the solid symbols. Squares represent males and circles represent females. The animal number is given above each symbol; the numbers below the solid symbols indicate weight ratios at 9 weeks old compared with age- and sex-matched littermates. *a*, Pedigree of MT-GRF mouse 7672-5; *b*, that of MT-GRF mouse 7675-2. Mouse 7675-2-6 died but was previously scored as a non-carrier.

To determine whether the MT-GRF fusion gene and the large size are heritable, two of the MT-GRF founder animals were bred with control animals; offspring from these matings were analysed for the presence of the MT-GRF fusion gene and for their rate of growth. Figure 2 shows two pedigrees demonstrating that both the fusion gene and the 'large' phenotype are inherited by ~50% of the total offspring. DNA analysis showed that all positive offspring carried the same number of copies of the fusion gene as the corresponding parent (data not shown). Four other females were also mated and produced litters. Thus, all six of the females tested were fertile (two were killed before breeding). In addition, male MT-GRF mice 760-5 and 801-9 (see Table 1) sired litters that included offspring carrying the fusion gene and displaying the large phenotype, indicating that both males and females carrying the MT-GRF fusion gene are fertile and transmit the gene. We now know that both the MT-GRF fusion gene and the large phenotype are inherited by ~50% of the offspring examined in the F_2 generation of animals.

To determine whether expression of the MT-GRF fusion gene in transgenic mice demonstrates a tissue specificity similar to that of the endogenous MT-I gene, we analysed both MT-I and MT-GRF RNAs in six tissues from either a control mouse or an MT-GRF mouse (female 765-2-3) (see F_1 in Fig. 2*b*); the

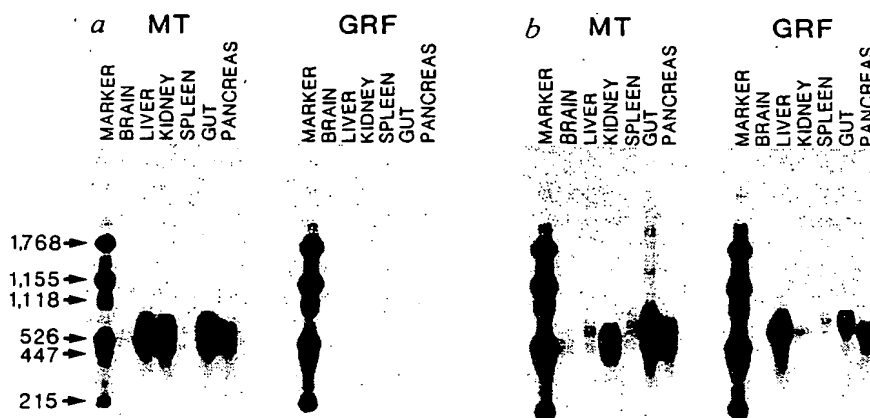
results are presented in Fig. 3. The control animal expressed MT-I mRNA at high levels in liver, kidney, gut and pancreas, and at lower levels in brain and spleen. This agrees well with the known tissue distribution of MT-I mRNA¹⁸. The control animal did not express detectable hGRF mRNA in any tissue. Although the hGRF probe might detect endogenous mouse GRF mRNA in the brain, the low abundance of GRF mRNA even in the hypothalamus (<0.01%) makes detection unlikely. The transgenic mouse 765-2-3 showed a tissue distribution of MT-I mRNA much like that of the control mouse with one exception; the amount of liver MT-I mRNA was substantially reduced (Fig. 3). The reason for this is unclear, but it cannot be explained by RNA degradation and is a reproducible result in this animal. In the transgenic mouse, the MT-GRF fusion mRNA is expressed at high levels in the liver, gut and pancreas, at low levels in kidney and spleen, and was not detected in the brain (Fig. 3). Although the expression of the fusion gene in this animal demonstrates a tissue specificity similar to that of the mouse MT-I gene, there are clear and reproducible differences, most noticeably the low level of expression of MT-GRF mRNA in the kidney. We have recently examined the pattern of MT-GRF gene expression in a second pedigree (801-5-9) and have found that although the general pattern of tissue specificity parallels that of mouse MT-I, there are subtle differences particular to this animal and distinct from the MT-GRF animal 765-2-3 (data not shown). Similar results were obtained on examination of the tissue specificity of metallothionein/growth hormone fusion genes¹⁵. In this case, an extensive survey of multiple animals showed that expression varied among different tissues and different animals, suggesting that factors such as the site of integration probably influence expression of the foreign gene.

Although many tissues apparently express the MT-GRF mRNA and presumably synthesize the GRF precursor protein, we do not know which tissues are capable of proteolytically processing this precursor to generate the mature GRF peptide. The antibody used to detect plasma GRF would detect both the precursor and the mature peptide¹⁰. It seems likely that such processing must occur to some extent, as the free N-terminus of the GRF peptide is known to be required for biological activity^{7,8}. We are presently using chromatographic techniques to determine what percentage of the immunoassayable GRF in the plasma is mature peptide as opposed to precursor.

Our results demonstrate that expression of supraphysiological levels of GRF in mice leads to increased somatic growth. Conversely, it has been shown that passive immunization of rats with antibodies against GRF can inhibit somatic growth²⁵. Taken

Fig. 3 Expression of human GRF mRNA in MT-GRF transgenic mice. *a*, Results from a control mouse; *b*, results from MT-GRF transgenic mouse 765-2-3. Each panel shows Northern blots of RNAs isolated from six different tissues. Duplicate blots from each animal were probed with either mouse MT-I (mMT-I) or hGRF probes as indicated. Size markers (in nucleotides) are denatured *Hind*III-cut simian virus 40 DNA.

Methods. Both animals were maintained on water with 76 mM $ZnSO_4$ for several weeks before killing. The indicated organs were removed and frozen at $-70^\circ C$ until use. Total RNA was prepared by homogenization in guanidine isothiocyanate and centrifugation through caesium chloride as described²⁹. 5 μg of each RNA was denatured and electrophoresed on formaldehyde/1.5% agarose gels³⁰ and the RNA transferred to nitrocellulose filters as described³¹. Filters were probed with either a mouse MT-I genomic clone, pSH¹³, or a human GRF cDNA clone, phGRF-54 (ref. 10). Probes were labelled by nick-translation using [α -³²P]dCTP (NEN). Exposure time of the autoradiograms ~16 h.



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together, these data suggest strongly that GRF has an important role in the promotion of growth. Transgenic mice carrying the MT-GRF fusion gene have elevated levels of plasma growth hormone and grow at a rate 25–50% greater than that of control littermates; this is significantly less than the increased growth observed in transgenic mice expressing growth hormone, many of which grow to twice the normal size^{14,15}. However, in the transgenic animals expressing growth hormone, most body tissues express the gene and have the potential to make growth hormone, whereas in our experiments growth hormone is only made in the anterior pituitary and thus may be rate-limiting. An unexpected finding was that females expressing the MT-GRF fusion gene are fertile, although transgenic female mice expressing the growth hormone gene are generally infertile¹⁶; this suggests that the enhancement of growth in the MT-GRF females is more physiological, perhaps because the effect is mediated through endogenous somatotropes.

We examined the pituitaries of two MT-GRF transgenic animals that express the fusion gene at high levels (9762-5 and 9765-2-3). In both cases the pituitary was at least three times normal size and showed signs of marked hyperplasia. The condition of these animals is remarkably similar to the clinical findings in patients with certain types of human growth disorders characterized by elevated levels of growth hormone in serum and pituitary hyperplasia. Rather than pituitary adenoma, the more common cause of such symptoms, a tumour is found at a remote site, often a pancreatic or bronchial carcinoma^{26–28}. These tumours do not produce growth hormone, yet on resection plasma GH levels fall. Such rare tumours are now recognized to be ectopic sources of GRF^{7,8} and the MT-GRF transgenic mice seem to provide a good animal model for this class of growth disorders. The finding of pituitary hyperplasia suggests that GRF, in addition to regulating GH production, might have a role in somatotroph proliferation.

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Phagosome acidification blocked by intracellular *Toxoplasma gondii*

L. David Sibley*, Earl Weidner* & James L. Krahenbuhl†

* Department of Zoology and Physiology, Louisiana State University, Baton Rouge, Louisiana 70803, USA

† Department of Immunology Research, National Hansen's Disease Center, Carville, Louisiana 70721, USA

Toxoplasma gondii belongs to a group of highly virulent intracellular parasites that reside in host cell vacuoles which resist typical phagosome-lysosome fusion¹. Live *Toxoplasma* replicate prodigiously within modified phagocytic vacuoles formed during invagination of the host plasma membrane^{2,3}. In contrast, heat-killed *Toxoplasma* or specific antibody (heat-inactivated)-coated live *Toxoplasma*-containing vacuoles readily undergo lysosome fusion and digestion in normal macrophages^{2,4}. Of newly recognized significance to *Toxoplasma* survival is the microbicidal effect of phagosome acidification, which reportedly can occur independently of fusion with other acidic vesicles^{5–8}. We report here that modified live *Toxoplasma*-containing vacuoles fail to acidify in normal macrophages, as indicated by the sensitive pH probe fluorescein. In contrast, when live *Toxoplasma* are coated with specific antibody (heat-inactivated), they trigger phagosome acidification when entering normal macrophages. A similar acidification is observed when normal phagocytes ingest dead *Toxoplasma*. Extracellular *Toxoplasma* are highly susceptible to acidic pH conditions, indicating that the acidification block in the modified vacuoles may be important for intracellular survival.

The endogenous acidification of macrophage phagosomes may involve two independent mechanisms that operate before acidic vesicle fusion. First, NADPH-oxidase activity, associated with the phagocytic respiratory burst, forms superoxide radicals and delivers H⁺ ions to the phagosome⁹. Second, phagosome acidification may involve the Mg-ATPase H⁺-ion pump identified in pinosomes⁹ and ligand-receptor endosomes^{10–15} also formed by plasma membrane invagination. Given the potential for similar acidic changes in newly formed phagosomes⁶ and the pH susceptibility of *Toxoplasma*^{16,17}, we investigated how the *in situ* pH of macrophage vacuoles containing *Toxoplasma* affected intracellular survival.

The fate of live compared with heat-killed or antibody-coated *Toxoplasma* in normal or activated mouse peritoneal macrophages was evaluated microscopically at intervals after infection *in vitro*. Live *Toxoplasma* here refers to extracellular parasites freshly collected from mouse ascites¹⁸, representing the maximum viability obtainable. Acridine orange was used to prelabel acidic cell compartments, including lysosomes of macrophages cultured on coverslips¹⁹. One hour after infection, 80% of live *Toxoplasma*-containing vacuoles blocked fusion of acridine orange-labelled vesicles in normal macrophages. However, 86% of heat-killed (60°C for 10 min) or antibody (heat-inactivated)-coated live *Toxoplasma* vacuoles fused with acridine orange-labelled vesicles within 1 h of phagocytosis by normal macrophages. In activated macrophages from immune mice⁹ which were cultured *in vitro* for 8 h, 85% of live *Toxoplasma* vacuoles fused with acridine orange compartments, were digested, and thus resemble heat-killed or antibody-coated *Toxoplasma* vacuoles.

To evaluate the cidal effect of acidic pH, extracellular *Toxoplasma* were incubated in media at fixed pH values and then evaluated for their ability to survive in host cells. The viability of live *Toxoplasma* was reduced to ≤60% of control after incubation in media at pH 6.0, as evaluated by plaque formation on fibroblast monolayers²⁰ and fusion with acridine orange-labelled vesicles in normal macrophages (Fig. 1).

Having established the high susceptibility of extracellular *Toxoplasma* to acidic pH, we examined the *in situ* pH of modified